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Keywords Equine primary melanoma cultures · Characterization · Melan-A · MAGE-1 · MAGE-3 · Melanoma-associated antigen

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Introduction

Gray horses frequently develop cutaneous melanomas which eventually metastasize, especially during the second half of their life. It is estimated that up to 80% of gray horses older that 15 y develop melanoma (McFadyean 1933; Rodríguez et al. 1997). Equine melanomas are classified according to their clinical patterns as melanocytic nevi (melanocytoma), dermal melanoma, dermal melanomatosis (multiple, often confluent dermal melanomas) or anaplastic malignant melanoma. Melanocytic nevi are encountered in younger horses of all colors, and are discrete, located in the superficial dermis or dermo-epidermal junction and are benign. Dermal melanomas are located in the deep dermis. Anaplastic malignant melanomas

are in contrast to melanocytic nevi and dermal melanomas highly polymorphic, often poorly pigmented and exhibit a high mitotic rate. Equine dermal melanoma in gray horses mainly develop under the tail root, in the anal, perianal and genital regions, perineum, lips and eyelids as nodules with a low tendency to metastasis in contrast to equine dermal melanomatosis which have high incidence of metastasis. Both types of melanoma are hard to distinguish from each other and, to date, their metastatic potential cannot be determined by histological analysis (Valentine 1995; Seltenhammer et al. 2004).

Diagnosis of equine melanoma is based on clinical and histopathological examination including cytology. Often, melanomas are not treated because of their initial benign behavior, slow-growth, proximity to major vessels and vital structures, or extensive local invasion (MacGillivray et al. 2002). There also may be increased risk of metastasis after mechanical stimulation of a melanoma. Treatments include surgical excision, partial tail amputation, cryotherapy, oral systemic therapy with cimetidine, and intralesional chemotherapy with cisplatin (Frederick 1990; Goetz et al. 1990; Goetz and Long 1993; Théon et al. 1993; Warnick et al. 1995; Freeman 1999; Rowe and Sullins 2004). Except for small and well-demarcated melanomas, surgical excision has poor prognosis (Cox et al. 1989).

Recently, research on treatment of human and animal melanomas has focused on immunoprophylaxis. A crude whole-cell melanoma vaccine has been used with non-conclusive results in the management of equine neoplasia (Jeglum 1999; MacGillivray et al. 2002). Intratumoral injection of interleukin-18 and interleukin-12 DNAs showed some inhibitory effect on melanoma growth in gray horses (Heinzerling et al. 2001; Stähli 2005). However, a vaccine resulting in complete remission is still to be developed.

New therapeutic approaches for treatment of equine melanoma are therefore required. For this to be achieved, preclinical models need to be established to allow the determination of the efficacy of new therapeutic approaches. This include the use of in vitro cultured melanoma cells, which would offer various advantages, such as being derived in large quantities and being easily accessible for drugs and vaccine testing. Unlike in other species, the establishment of primary equine melanoma cultures has remained a major challenge. Additionally, a reliable marker system for equine melanomas still remains to be established (Seltenhammer et al. 2004).

The objective of this study was to establish a method for routine isolation and cultivation of primary equine melanoma cell cultures. To ensure that the cultures isolated were melanoma cells, the presence of some of the known melanoma-associated antigens (MAA) was determined. These included; Melan-A, MAGE-1, and MAGE-3. Melan-A is a melanocyte differentiation antigen, recognized by autologous cytotoxic T lymphocytes. Melan-A is also known as MART-1 (melanoma antigen recognized by T cells 1). Currently, little is known about the function of Melan-A in melanocyte development and differentiation (Koenig et al. 2001; Murer et al. 2004; van Dinten et al. 2005). Melan-A/MART is expressed in normal immature melanocytes and in the majority (more that 90%) of fresh melanoma tumors and melanoma cell lines, but it is not expressed in other cells and other tumors. Because of the high percentage of melanoma tumors showing Melan-A expression and due to its immunogenicity, this antigen is one of the targets for cellular immunotherapy against malignant melanoma. Its expression can be used as a predictive factor for the selection of patients eligible for Melan-A-based immunotherapy (van Dinten et al. 2005).

The second group of antigens includes melanoma antigen-encoding (MAGE) family members which are normally expressed in adult testis. MAGE family of proteins are also expressed in various tumor types but silenced in normal adult tissue. MAGE-1 and MAGE-3 have been detected in melanoma cells and their ectopic expression is characteristic of malignant transformations (Urosevic et al. 2005). These antigens are presented in HLA-class-1-restricted fashion and can be recognized by antigen specific cytolytic T lymphocytes (CTL), which can eliminate melanoma cells upon recognition of these antigens (Boon and van der Bruggen 1996). The efficiency of this antigen presentation may depend on both melanoma- and host-immune specific factors. Melanoma-specific factors encompass the levels of MAA expression as well as the level of HLA class1 molecule expression (Urosevic et al. 2005). In addition to the above markers, the expression of proliferating cell nuclear antigen (PCNA) was determined. PCNA had previously been correlated with aggressive behavior in human cutaneous malignant melanomas (Ben-Izhak et al. 2002).

In this study, we established the isolation and cultivation of primary equine melanoma cells from excised melanoma tissue. We investigate the expression of several MAA—Melan-A, MAGE-1, MAGE-3, and PCNA, by means of Western blotting, FACS analysis, and confocal microscopy.

Materials and Methods

Excision of melanoma tissue. Melanomas were excised from 13 healthy gray horses (Table 1). The horses were placed in stalls and sedated intravenously with 0.01 mg/kg detomidine (Domosedan®—Pfizer AG, Zurich, Switzerland) and 0.02 mg/kg butorphanol (Morphasol®, Dr. E. Graeub AG, Bern, Switzerland). This was followed by a continuous rate infusion of detomidine and the flow was adjusted when needed. Just before surgery, 1 mg/kg flunixin

Table 1. Characteristics of excised melanoma tissue and presence or absence of growth after isolation

Melanoma	Age (y)	Sex	Breed	Site	Result
M1	16	mc	Irish WB	Head	G
M2	17	mc	Swiss WB	Pectoral	NG
M4	20	mc	Anglo-Arabian	Perianal	G
M8	10	mc	Lusitano	Perianal	G
M9	14	f	Arabian	Perianal	G
M10	12	mc	Swiss WB	Perianal	NG
M11	9	mc	Durch WB	Head	NG
M12	12	mc	Swiss WB	Perianal	G/NG[a]
M13	26	mc	Andalusian	Perianal	NG
M14	12	mc	Andalusian	Perianal	G
M15	8	f	Swiss WB	Dorsal tail	NG
M16	6	m	Andalusian	Perianal	G
M17	9	f	Arabian	Ventral tail	G

m Male intact, *mc* male castrated, *f* female, *ND* histological examination not done, *WB* warmblood, *G* cell growth, *NG* no cell growth
[a] Initial growth followed by cell necrosis after first cell division

meglumine (Fluniximin®, Berna AG, Bern, Switzerland) was administered intravenously. Tumors that were selected had an average diameter size of 4 to 20 mm, were easily accessible and the excision site chosen had low risk of wound-healing complications. The hair was clipped and the surgical site prepared aseptically. Lidocaine 2% (G. Streuli & Co. AG, Uznach, Switzerland) was used as the local anesthesia and it was administered subcutaneously on the perimeter of the tumor. The melanoma was excised according to standard surgical methods. The volume of the excised tumor material was estimated. Immediately after excision, the tumor tissue was divided in two pieces and one piece was placed in 4% PBS-buffered formalin (pH 7) for histological examination, while the other piece was placed in sterile Dulbecco's phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA) containing 1% Penicillin–streptomycin (Pen–Strep, Invitrogen) at room temperature, for isolation of melanoma cell cultures. It was immediately transferred to the cell culture laboratory. Time from excision to further processing of the cells was 15 to 20 min for all excised tumors.

Histological examination. Fixed tissue was embedded in paraffin and 4-μm sections were cut and stained with hematoxylin and eosin. Histological sections were evaluated for the following features: location of the tumor (e.g., superficial or deep dermis), presence of junctional activity, type of neoplastic cells (e.g., round, epitheloid, spindle-shaped), degree of pigmentation, degree of polymorphism, mitotic activity, presence of inflammation or necrosis within the tumor, growth pattern (e.g., expansive or infiltrative), lesions in the adjacent tissue (e.g., ulceration of the epidermis, dermatitis). No classification into benign and malignant melanomas was made as there are no reliable criteria to differentiate them on a histological basis. However, it should be noted that about two-thirds of the equine melanomas are thought to undergo progression and be capable of metastasis (Scott and Miller 2003; Seltenhammer et al. 2004).

Isolation of primary equine melanoma cells. The excised melanoma tissue was washed with PBS containing 1% Pen–Strep to get rid of blood. All the connective tissue surrounding the melanoma tissue was excised. The melanoma tissue was minced with scalpel blade into 1–2-mm pieces and washed again with PBS. Samples were subsequently digested with dispase (Boehringer Mannheim, Mannheim, Germany) diluted 1:2 in RPMI 1640 (Gibco, Carlsbad, CA), for 3 h at 37°C. After centrifugation at 1,500 rpm for 5 min, the supernatant was discarded and collagenase (Sigma, St. Louis, MO) diluted 1:100 in Tris-buffered saline (TBS) containing 10 mM $CaCl_2$, was added and mixture incubated for 2 h at 37°C. The reaction was stopped by the addition of 1/10 vol. of TNE buffer (0.05 M Tris base, 0.15 M NaCl, 0.01 M EDTA, pH 7.4) for 5–10 min. The cells were subsequently washed twice with RPMI 1640 medium containing 10% FCS and 1% Pen–Strep. Detached cells were separated from undigested melanoma tissue by filtration through sterile 70-μm nylon cell strainers (Falcon, Bedford, MA), and filtered cells were subsequently cultivated at 37°C and 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen), 5 mM glutamine (Invitrogen), 1 mM sodium pyruvate (Gibco) and 1% of an antibiotic mixture containing 10,000 U/ml penicillin and 10,000 μg/ml streptomycin (Gibco).

All cultures were examined daily for growth, adherence, and presence of contamination using an inverted light microscope. Media was replaced once per week and the first passage done once the cells reached >90% confluence.

A 1:2 dilution was used for slow-proliferating cells and 1:3 dilution for fast-proliferating cells. Cells that grew after isolation until the first passage were passage 0 (P0) cells, and after the first passage they were P1, and this applied for the subsequent passages. For our experiments we used melanoma isolates in passages P0–P3.

To ensure that the melanocytes were not contaminated with fibroblasts, one plate of the P1 cells from the fast-proliferating M9 and M14 isolated were treated with 100 μg/ml geneticin (G418), which is known to completely inhibit fibroblast growth in melanocyte isolates (Halaban and Alfano 1984).

For the cell passage, confluent adhering cells were detached with a solution containing 0.05% trypsin and 0.02% ethylene diamine tetra-acetic acid (EDTA; Invitrogen), inactivated with fresh media containing 10% FCS. Some of the detached cells were used to make stabilate stocks for storage in liquid nitrogen. After inactivation of trypsin, these cells were resuspended in Recovery™ cell culture freezing medium (Invitrogen) and cells were placed in Nalgene™ Cryo 1° Freezing container, which had isopropanol insulation. They were stored at −80°C overnight and then transferred to liquid nitrogen.

An established equine melanoma cell line (kindly provided by Dr. M. Seltenhammer, University of Veterinary Medicine, Vienna, Austria) was used as a positive control and was grown under the same conditions as the isolated primary melanoma cells. This positive control cell line had been in culture and had undergone several passages; the cells we used were after passage 30.

Primary equine muscle fibroblasts (passage 2) were used as negative control cells. Muscle tissue (≈0.5 cm) was excised from the lower abdomen of a live horse and primary fibroblasts were isolated using sequential digestion with dispase and collagenase similar to the melanoma cells, as stated above. The isolated cells were then propagated in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and 1% of an antibiotic mixture containing 10,000 U/ml penicillin and 10,000 μg/ml streptomycin (Gibco).

Antibodies. The mouse monoclonal Melan-A antibody with specific reactivity for human and horse melanomas was purchased from Biomeda AG, Zurich, Switzerland, while the mouse polyclonal MAGE-1 and MAGE-3 antibodies were a gift from Prof. Giulio C. Spagnoli, University of Basel. Mouse Monoclonal anti-PCNA and anti-tubulin antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. The tubulin antibody was used as a loading control.

FACS analysis for melanoma cells. For FACS analysis, the procedures described by Urosevic et al. (2005) were used with some modifications. In brief, passage 2 cells were harvested by treatment with trypsin, washed once with medium containing 10% FCS. The cells were subsequently washed once with F-PBS (phosphate-buffered saline without Ca^{2+}/Mg^{2+} containing 1% FCS and 0.02% sodium azide). Cells were then fixed with 4% paraformaldehyde for 20 min, at room temperature. This was followed by permeabilization with PBS containing 0.2% Triton X-100 for 10 min at room temperature. The cells were then washed twice with F-PBS and incubated with primary antibody against respective MAA, at room temperature for 1 h on a roller (200 μl PBS containing 2% BSA, 0.1% Triton X-100). The following antibody dilutions were used; Melan-A (1:100), 77B (MAGE-1; 1:25) and 57B (MAGE-3; 1:25). The cells were then washed twice with F-PBS and incubate with secondary anti-mouse FITC conjugated antibody (1:250) in 200 μl PBS containing 2% BSA, 0.1% Triton X-100 on ice in the dark for 1 h. Anti-mouse antibody not conjugated to a fluorescent dye was used as negative control. Cells were washed twice with F-PBS and incubated in 0.5% paraformaldehyde for 5 min. For FACS analysis, at least 10,000 cells were acquired and fluorescence detected using Beckman Coulter FC500 cytometer and analyzed using a CXP analysis software.

Immunofluorescent labeling. The melanoma cells after one passage were seeded onto four-chamber slides (Lab-Tek®, Nalge Nunc International, Rochester, NY) and allowed to grow until they reached an 80% confluence which was achieved within 3 d after seeding. The medium was aspirated, cells washed twice with PBS before fixation with 4% paraformadehyde for 20 min at room temperature. Cells were then permeabilized in PBS containing 0.2% Triton X-100 for 10 min. Cells were washed twice in PBS and subsequently blocked in PBS containing 2% BSA and 0.1% Triton X-100 for 45 min at room temperature.

The cells were subsequently incubated with primary antibodies; Melan-A (1:100), MAGE-1 (1:25), and MAGE-3 (1:25) dissolved in PBS containing 2% BSA and 0.1% Triton X-100, for 1 h at room temperature. Cells were then subsequently washed twice with PBS.

Secondary FITC-labeled anti-mouse antibody (1:250) dissolved in PBS containing 2% BSA and 0.1% Triton X-100 was then added and the slides completely covered with aluminum foil and incubated for 90 min at room temperature. From this point onwards, all subsequent steps were done in the dark to avoid bleaching of the fluorescent probes. The cells were then washed once in PBS before 300 μl of DAPI (0.1 μg/ml) was added and incubation done for 5–10 min. The cells were washed twice with PBS.

The chambers were torn away and the rubber at the chamber boundaries removed carefully to avoid damaging of the cell monolayer. One to two drops of mounting

solution (Vector Shield) was added on to cells of each well, and cells were subsequently covered with cover slips and sealed with transparent nail polish. Slides were protected from light and stored at 4°C until examined by confocal microscopy.

SDS-PAGE and immunoblotting. Melanoma cell cultures were washed twice with PBS containing protease inhibitors. The cells were then scrapped off in cold PBS containing protease inhibitors. After centrifugation, the cell pellet was lysed and samples were analyzed immediately or stored at −80°C.

The protein concentrations were determined with the Bio-Rad D_C protein assay before analysis by SDS-PAGE and immunoblotting. Samples were denatured by heating for 5 min at 95°C, before being loaded onto a 10% (*w*/*v*) gel for MAGE-1, MAGE-3, PCNA, and tubulin detection (20 μg protein per slot) and onto a 20% (*w*/*v*) gel for Melan-A detection (20 μg protein per slot). After separation by electrophoresis, the proteins were transferred electrophoretically onto hybond-C nitrocellulose membranes, which were blocked for 1 h at room temperature in Tris-buffered saline (TBS; 500 mM NaCl, 9 mM Tris–HCl, pH 7.4) and 0.1% Tween 20 (TBST), containing 5% (*w*/*v*) skimmed milk powder. The antibody labeling was done in TBST containing 1% (*w*/*v*) skimmed milk powder for 2 h at room temperature for polyclonal mouse anti-MAGE-1 (1:50), MAGE-3 (1:50), PCNA (1:1,000), and tubulin (1:1,000), while for Melan-A, the labeling was done for 12 h at 4°C with the monoclonal mouse anti-equine Melan-A antibody (1:200). The nitrocellulose membranes were washed three times in TBST containing 1% (*w*/*v*) skimmed milk powder for 45 min at 4°C, and incubated with the anti-mouse (1:3,000) secondary antibody conjugated with alkaline phosphatase in the same buffer for 2 h at room temperature. They were analyzed with an enhanced chemiluminescence immunoblotting detection system.

Results

Macroscopic and histological findings. Six of the eight primary melanoma cell cultures that grew in vitro were from tumors that were excised mainly from the perianal region, while the other two were from the head, and the ventral base of the tail (Table 1). These tumors were located intradermally. The melanoma tissue consisted of a black pigmented solid mass that was easy to differentiate from normal tissue. The consistency of the tumor mass differed, some being very firm and resistant to dissection while the others were soft and easy to dissect. In some melanomas, the center was composed of dark pigmented material that was gritty and it disintegrated easily when touched. The shapes of the tumors were round, oval, or irregular.

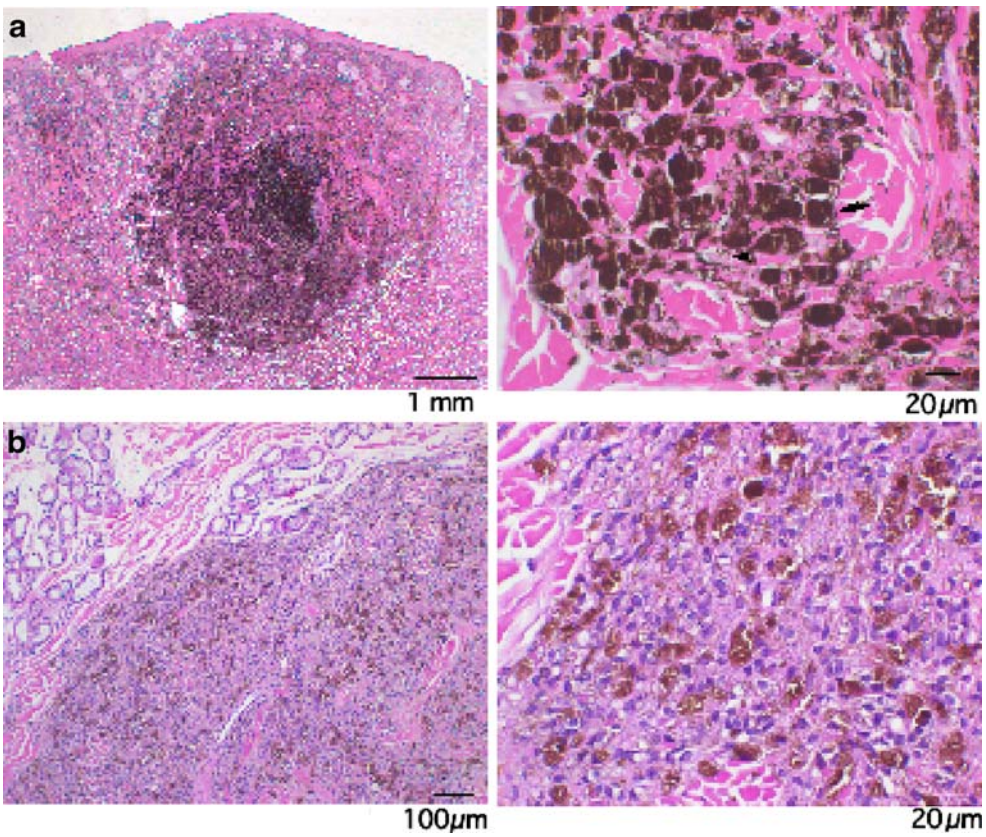

Figure 1. Hematoxylin and eosin staining of melanoma tissues excised from dermally located melanoma M4 (*a*) and M9 (*b*). M4 (*a*) was pigmented, non-encapsulated and had infiltrative growth. It was composed of medium to large sized, round, polyhedral (*arrow*) or plump-spindled (*arrowhead*) cells that contained large amounts of melanin pigment. M9 (*b*) was moderately pigmented, well circumscribed, with mild infiltrative growth. It was densely packed with spindle-shaped cells with ovoid nucleoli with low to moderate amounts of melanin pigment.

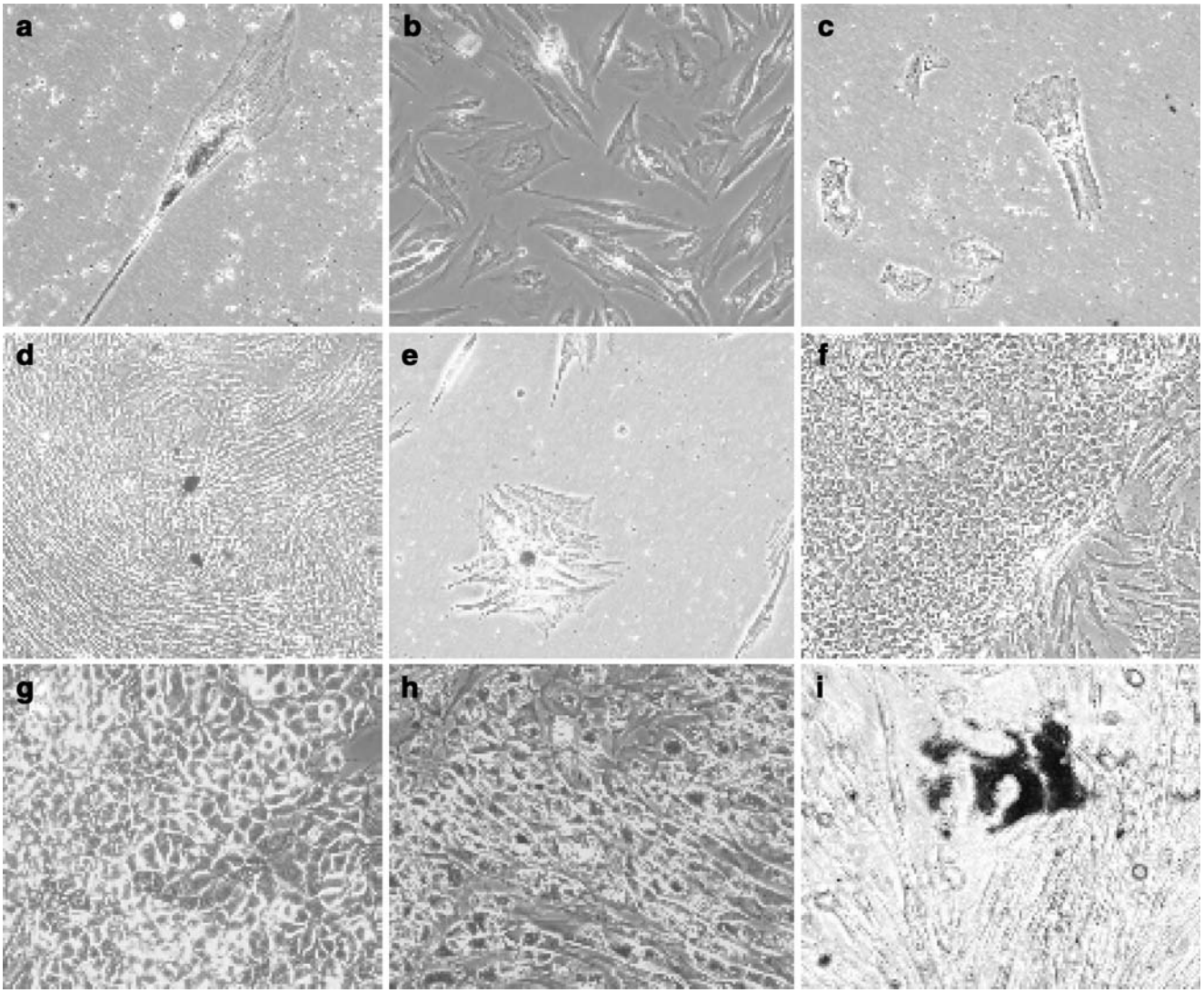

Figure 2. Phase contrast microscopy of cells isolated from melanoma tissues of the following melanoma; M8 (*a*)—*P0*, M17 (*b*)—*P1*, M12 (*c*)—*P0*, M14 (*d*, *h*, *i*)—*P1*, M1 (*e*)—*P1*, M9 (*f*)—*P1* and positive control (*g*)—*P30*. The passage numbers are represented in *italics*.

All melanocytic tumors were dermally located, non-encapsulated, infiltratively growing, and usually composed of a mixed population of spindle, round, and polygonal neoplastic cells (Fig. 1*a* and *b*) with a variable number of admixed melanophages. No anaplastic melanoma was observed. The degree of pigmentation was variable, but usually high and no amelanotic tumor was seen. The nuclear shape varied and was either ovoid or round, the chromatin was mostly finely stripped and one, or in rare cases, two small but distinct nucleoli were present. The degree of anisokaryosis and ansiocytosis was mild to moderate and mitotic figures were not observed. Some tumors were in close proximity to the epidermis; however, junctional activity was not present. Small perivascular nests of tumor cells were present in M4, and M12 and, in two cases (M4 and M14), a tumor embolus within a vessel was detected. In five cases (M4, M8, M13, M15, M16), small, only histologically detectable periadnexal neoplastic proliferations were present in addition to the main tumor mass. A predominantly lymphocytic inflammatory reaction, mostly confined to the border of the tumor, was present in one case (M15) and a necrotic center with dystrophic mineralization in another (M2). From 13 excised melanomas, five did not grow in culture. However, histologically, no difference was detected between these five and the isolates that successfully grew in culture.

Melanoma cell cultures. Out of the 13 excised melanoma tissues, we succeeded in establishing eight melanoma primary cell cultures, which corresponded to a success rate of 62%. It took a minimum of 7 d for adherent cell colonies to be detected on the culture dishes and 14–21 d to begin to proliferate. The shape of the isolated melanoma cells differed ranging from bipolar and slim cells, tripolar cells,

cells with polydendrites to rounded cells (Fig. 2*a*,*b*, and *c*). Some of the cultures grew as a uniform monolayer (Fig. 2*d*) while others accumulated as clusters of cells with pigmentation at the center (Fig. 2*e*).

Most cultures proliferated slowly and required about 7 d to reach confluence after a 1:2 dilution. In contrast, two cultures, M9 and M14, required 3 d to reach confluence after a dilution of 1:3. M9 had two distinct cell populations, one being a very fast-proliferating rounded cell population with some cells having long dendrites extending to the next rounded colony. These populations were surrounded by slow-proliferating bipolar and tripolar spindle-shaped cells (Fig. 2*f*). Pigmentation in these cells was low. The shape of the rounded cells was similar to our positive control cells that were highly proliferating and had similar dendritic extensions to the next rounded colony (Fig. 2*g*). There was no difference in cell properties or proliferation of the M9 and M14 cells that were treated with 100 μg/ml genetic (G418), which indicated that our cells were not contaminated with fibroblasts.

Pigmentation in the different cultures showed variations, ranging from low, intermediate to heavily pigmented cells. In some bipolar and tripolar cells, pigmentation was found at the center of the cell in the perinuclear area (Fig. 2*a*) or in the middle of the cluster of several cells (Fig. 2*e*). In one culture (M12), very few melanocytes attached to the plate surface. These cells were larger than in the other cultures and they had pigmentation in the perinuclear regions (Fig 2*c*). These cells died off after the first division. In M14 culture, a lot of cells in several large sections of the monolayer had heavy pigmentation and there were also several huge irregular cells filled up with heavily pigmented granules (Fig. 2*h* and *i*). After a few days, the heavily pigmented cells became necrotic and detached from the plate surface.

After the first passage, cells required less time to reach confluence, on average, one to two weeks. The proportion of cells having clear pigmentation by the time confluence was reached decreased although pigmentation increased upon longer cultivation, e.g., after 2 wk. The cells were maintained in culture for a maximum of three passages. After this, cell stabilates were prepared and stored in liquid nitrogen.

FACS analysis. FACS analysis proved to be sensitive in detection of expression of melanoma-associated antigens. More than 80% of the positive control cells expressed Melan-A, while in the cultivated isolates, the proportion of cells expressing Melan-A between 20% and 42%. Less than 10% of the cells in all the isolates including the positive control expressed either MAGE-1 or MAGE-3 (Fig. 3). The MAGE-1 and MAGE-3 signal were also low.

Confocal microscopy. To confirm the intracellular localization of the MAA, confocal microscopy was done. Fluorescent signals of several sections at different height of the sample were examined and results of the different z-sections shown. Our FACS analysis results were confirmed with confocal microscopy. All the positive control cells in the acquired microscopic field had very high cytoplasmic expression of Melan-A (Fig. 4*a* and *b*; *arrows*). In our isolates, there was expression of Melan-A, although only a few cells had distinctively high amounts of Melan-A (*arrows*). A proportion of the remaining cells showed Melan-A expression; however, the amount of protein was lower (Fig. 4*c–f*).

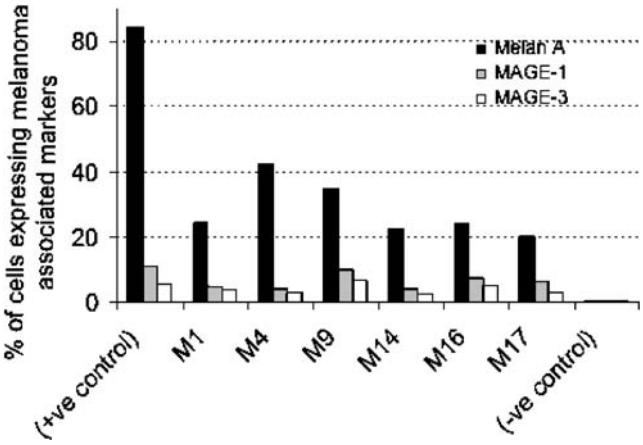

Figure 3. Expression of Melan-A, MAGE-1, and MAGE-3 detected by FACS analysis. Values are a mean of two experiments. The positive control cells were from passage 32 (P32), while the primary isolates were from second passage (P2).

In all the cell cultures, including the positive control cells, very few of the cells expressed MAGE-1 and MAGE-3 (*arrows*), and the amount of protein detected by confocal microscopy was low (Fig. 5*a–d*).

Western blotting. Western blotting was not as sensitive as the FACS and confocal microscopy in detecting the presence of MAAs. Several unspecific bands were detected using MAGE-1 and MAGE-3 polyclonal antibodies although none of these bands specifically corresponded with the molecular weights of MAGE-1 (46 kDA) or MAGE-3 (49 kDA). Using the Melan-A monoclonal antibody, high amounts of Melan-A (18–22 kDA) could only be detected in the positive control cell culture (Fig. 6). There was no signal from the other isolates using the normal ECL detection. When the more sensitive DURA was used, several unspecific bands appeared (data not shown). PCNA signal was detected in all the isolates, the signal was stronger in the positive control cells, the M9 and M14 isolates and the negative control muscle tissue cells. These cells were from fast-proliferating cell cultures. Tubulin was used as the positive control, and all the cultures had a similar signal (Fig. 6).

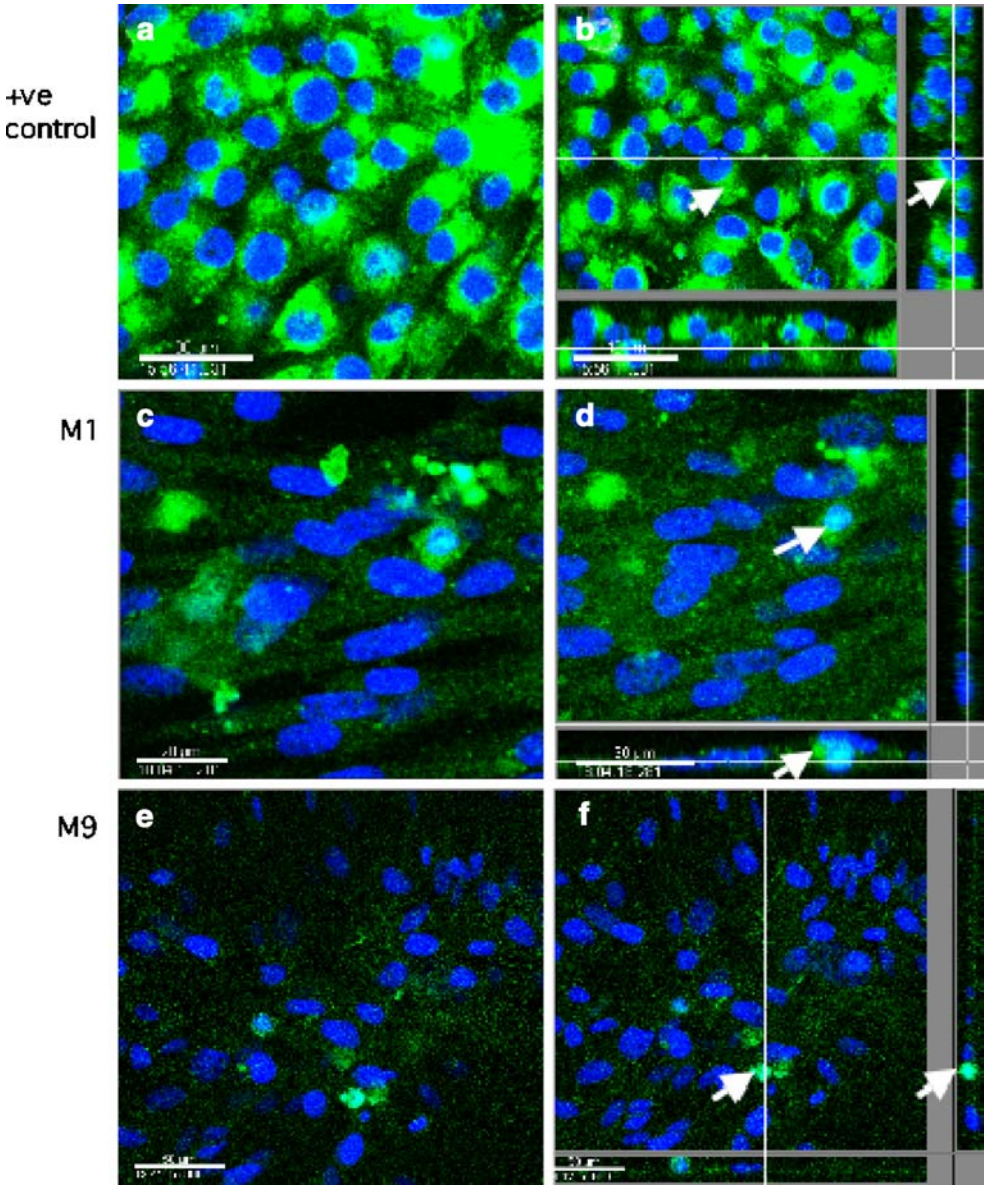

Figure 4. Confocal microscopy detection of the expression of Melan-A in melanoma cells; (*a* and *b*), positive control—*P32*; (*c* and *d*), M1-*P2*; (*e* and *f*) M9-*P2*. *Figures on the left* are *xy*-projections of chosen slices, while *figures on the right* show the *xz*- and *yz*-section projections with *arrows* showing the presence of clear intracellular staining. Passage numbers are in *italics*.

Discussion

We successfully developed and established a protocol for routine isolation and establishment of primary equine melanoma cells. Eight primary equine melanoma cell cultures out of 13 excised melanoma tissues grew in vitro, which corresponds to a success rate of 62%. The histological analysis confirmed that all the primary cultures that grew in vitro were excised from melanocytic tumors. According to our knowledge, this is the first study with the highest success rate in establishment and primary equine melanoma cell cultures.

Although histological examination showed no major differences between the tumors that grew and those that did not grow in culture, the hematoxylin and eosin staining used in our study did not differentiate between viable and necrotic cells in the melanoma tissues. We presume that the lack of growth in five melanoma tissue could be attributed to a low density of viable cells in the melanoma tissue.

All the primary cell cultures that adhered to the plates after isolation had pigmentation though the levels of pigmentation different. Variable pigmentation intensities were also previously reported in cultured primary human melanocytes (Hanke et al. 2005). The level of pigmentation was not as intense as in the excised tissue, and this can be attributed to the fact that melanoma cell cultures may rapidly alter mRNA and protein expression due to a lack of cell–cell interactions as previously indicated (Hofbauer et al. 2001). Loss of melanin granules in melanoma culture had also been reported previously in canine melanoma (Wolfe et al. 1987; Ritt et al. 1998; Koenig et al. 2001). However, it should be also taken into consideration that

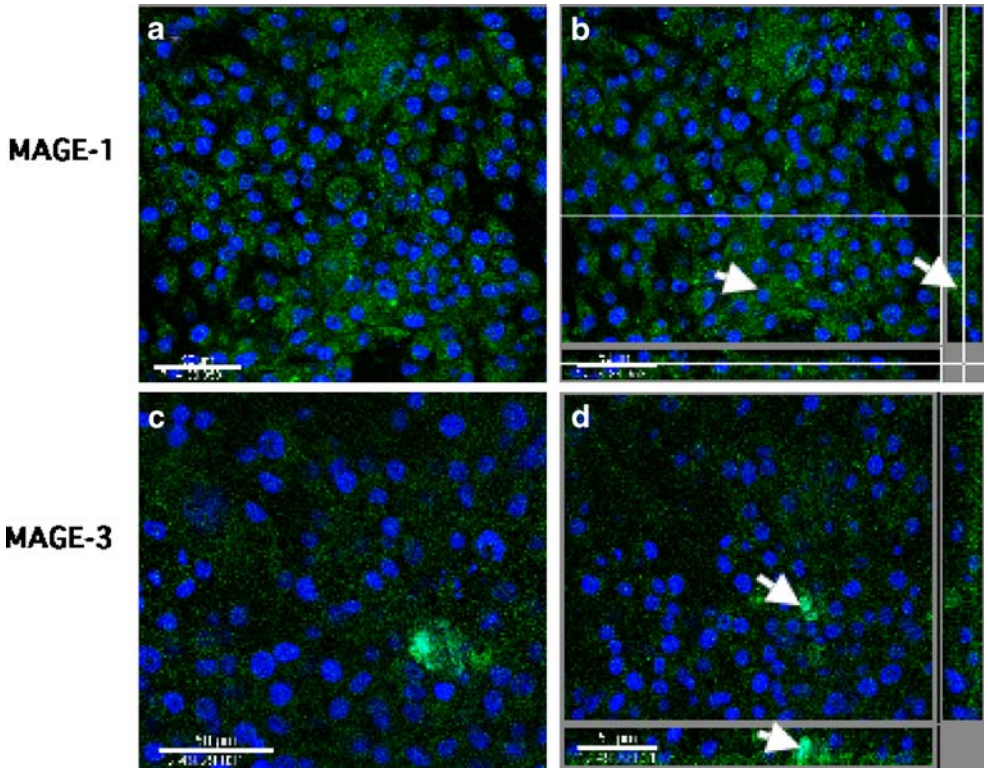

Figure 5. Confocal microscopy detection of the expression of MAGE-1 (*a* and *b*) and MAGE-3 (*c* and *d*), in melanoma positive control cells at passage 32 (P32), which is representative of the expression in the primary isolates. *Figures on the left* are *xy*-projection of chosen slices while *figures on the right* show the *xz*- and *yz*-section projections with *arrows* showing the presence of clear intracellular staining.

irregular intensities of pigmentation were also detected in the histology sections of the excised equine melanoma tissue. This was consistent with observations made in a previous study on equine melanoma tissue (Seltenhammer et al. 2004).

FACS analysis showed that more than 80% of the positive cell culture had the cells expressing Melan-A. These cells additionally had very high amounts of Melan-A detected by confocal microscopy. It is, therefore, not surprising that it is only in these cells that a positive Melan-A signal could be detected by western blotting. On the other hand, the proportion of cells expressing Melan-A in our isolated cultures, ranged from 20–42%, and the Melan-A signal in most of the cells was low. These differences could be attributed to the fact that the positive control cells have been in culture for extended periods and had been passaged more than 20 times. A previous study, too, had shown that canine melanoma cells that expressed Melan-A, were on passages above 50 (Koenig et al. 2001). Cells that have undergone several passages could have undergone in vitro selection resulting in expressing of high levels of Melan-A, since longer cultivation of melanoma cells has been shown to influence gene expression (Ritt et al. 2000). In contrast, the cells isolated in our study, were analyzed after one or two passages and we assume that at this point, no in vitro selection had occurred as yet. We purposely did not want to induce any in vitro selection that might influence the expression of genes that might be of interest later on in the study. However, despite this, the presence of cells expressing Melan-A confirmed that we had successfully established melanoma cell cultures since Melan-A is specific for melanoma cells and is not expressed in other cells or other tumors (van Dinten et al. 2005).

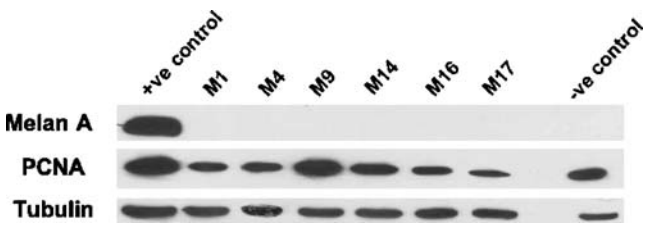

Figure 6. Expression of Melan-A and PCNA detected by western blot analysis. The positive control cells were from the 32nd passage (P32), while the primary isolates were from third passage (P3). Tubulin was used as a loading control.

Weak MAGE-1 and MAGE-3 signals could only be detected using the more sensitive FACS analysis and confocal microscopy. Using western blotting, MAGE-1 and MAGE-3 antibodies recognized several bands. Previous studies had also shown that the MAGE-3 antibody (57B) recognizes other members of the MAGE family, including MAGE-1, -4, -6, and -12 and, thus, regarded as a multi-MAGE antibody (Busam et al. 2000). However, none of the bands detected was strong enough for us to draw any conclusions on which MAGE proteins were highly expressed. It should be taken into account that MAGE

genes are absent in melanocytic nevi and malignant melanomas (Busam et al. 2000). Our analyses of histological sections and isolated cell cultures could not differentiate between malignant and benign melanoma, however, most of melanomas of gray horses eventually metastasize and two cases (M4 and M14) were presumably malignant with this assumption being strengthened by the presence of vascular invasion. We can, therefore, not rule out that malignancy was the cause of decreased or lack of clear expression of MAGE genes. On the other hand, in a previous study, MAGE antibodies had detected no signal with equine melanoma tissue (Heinzerling et al. 2001), hence, sensitivity of these antibodies to equine MAGE proteins should be studied.

PCNA is a 36-kDA molecule that plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery. It functions as the accessory protein for DNA polymerase δ, required for progressive chromosomal DNA synthesis in the S phase and it interacts with cellular proteins involved in cell-cycle regulation and checkpoint control (Kelman 1997). With this being taken into consideration, PCNA is, thus, not an exclusive marker for melanoma. However, higher expression of PCNA was previously correlated with aggressive clinical behavior in various tumors including cutaneous malignant melanomas in humans (Ben-Izhak et al. 2002). In our study, the expression of PCNA was higher in cells that rapidly proliferated in vitro including M14 that had vascular invasion and most likely malignant. However, more studies are required to confirm the malignancy and if there is any correlation between the level of PCNA expression and clinical behavior of equine melanoma.

Our study is, therefore, a step forward in providing an appropriate model for preclinical studies for development of potential treatment for melanomas. Horses spontaneously develop metastatic melanoma that resembles the human disease and primary equine melanoma cell cultures would be highly relevant for preclinical studies testing new immunotherapy protocols. The in-vitro-cultured equine melanoma cells would offer preclinical model to test the efficacy of potential therapies both for equine and human melanomas. Cultured melanoma cells would provide reliable and reproducible sources of tissue to develop and refine potential therapeutic approaches. They would also allow therapeutic manipulation of genes and proteins in living cells.

Previous studies had shown that intratumoral injection of interleukin-18 and interleukin-12 DNAs showed some inhibitory effect on melanoma growth in gray horses (Heinzerling et al. 2001; Stähli 2005). However, only a very low proportion of the tumors resolved completely, while the others just decreased in size without resolution. It remains to be determined if the lack of complete resolution was due to decreased DNA delivery to the melanoma cells or degradation of DNA before delivery. Having established primary equine melanoma cells would address this issue while allowing unlimited studies to be done to determine delivery of therapeutic gene in melanoma cells. This would give an insight on the most efficient gene delivery formulations and methods in these cells.

We were successful in the development and establishment of a protocol for routine isolation and cultivation of primary equine melanoma cells. The characterization of MAA is by no means exhaustive since we only used antibodies that were available in our institute at the point of isolation. To do a thorough characterization, more experiments will be required to determine the expression of other melanoma-associated antigens. Also, the effect of longer cultivation on gene expression will need to be determined.

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